ENDOTHELIAL FUNCTION

Circulating endothelial progenitor cells in patients with cardiac syndrome X

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Background: Cardiac syndrome X (CSX) encompasses the constellation of anginal chest pain in the presence of a pathological functional test and a normal coronary angiogram. Endothelial progenitor cells (EPCs) in the peripheral circulation contribute to tissue vascularisation.
Objective: To investigate the number and functional properties of circulating EPCs in patients with CSX.
Methods: 17 patients with CSX and a referent population (n = 20) were matched for age, atherosclerotic risk factors and use of drugs. Numbers of EPCs were studied by FACS, and their functional properties, including their proliferative capacity, adherence to matrix and mature endothelial cells as well as their ability to support in vitro tube formation, were investigated. Levels of soluble markers that associate with peripheral mobilisation and homing were studied in the serum samples of all subjects.
Results: Patients with CSX had significantly increased numbers of circulating EPCs as compared with the referent population (both CD34+/KDR and CD34+/CD133+). The proliferative capacity of EPCs and their ability to support in vitro tube formation were significantly impaired in patients with CSX as compared with the referent population. However, adhesiveness of EPCs from patients with CSX to fibronectin and cultured mature endothelial cells was enhanced as compared with the referent population. Serum vascular endothelial growth factor correlated with peripheral CD34+/KDR cell numbers, whereas serum concentration of erythropoietin correlated with the number of circulating CD34+/CD133+ cells
Conclusion: Patients with CSX have a significantly altered circulating EPC phenotype that could potentially aid in understanding the complex pathogenesis of the syndrome.

Cardiac syndrome X (CSX) is defined as chest pain in the presence of a pathological stress test or thallium scan and normal coronary arteries on angiography.[1 2] For several decades, CSX has represented a challenging diagnostic and therapeutic entity. Endothelial dysfunction with reduced coronary microvascular dilatory response and increased coronary resistance is thought to have an important role in the pathogenesis of CSX, with abnormal subendocardial perfusion detected by magnetic resonance imaging.[3] Endothelial dysfunction is characterised by a reduced bioavailability of endogenous nitric oxide and increased plasma levels of endothelin-1.[4 5] Other potential underlying mechanisms include abnormal pain perception, psychiatric disturbances, metabolic changes in the cardiac muscle, oestrogen deficiency, increased sympathetic tone and insulin resistance.[6]

Endothelial progenitor cells (EPCs) originate from the bone marrow and have the ability to proliferate, migrate and differentiate to mature endothelial cells.[7 8] Mobilisation of EPCs involves a series of events, including passage from the bone marrow, homing to areas of tissue damage and finally, angiogenesis and vasculogenesis. These process are governed by a complex network of endogenous cytokines and exogenous agents such as drugs.[9–11] Emerging data suggest that progenitor cells derived from circulating bone marrow have an important role in tissue regeneration throughout the cardiovascular system.[12 13] It has been shown that the number of circulating EPCs inversely correlates with risk factors for atherosclerosis, and reduced levels of EPCs are associated with endothelial dysfunction.[14 15] A decrease in circulating EPCs not only contributes to impaired angiogenesis but is also associated with accelerated atherosclerosis[16] and in-stent restenosis,[17] whereas ischaemia promotes mobilisation of EPCs to the peripheral blood.[18–20] Recently, therapeutic application of EPC transfer has been successfully implemented, with improvement of blood flow and left ventricular function in patients after myocardial infarction.[21]

Herein, we characterised the peripheral pool of EPCs in patients with CSX showing derangements that might be helpful in understanding the mechanisms leading to endothelial dysfunction present in this syndrome. Moreover, we studied systemic levels of factors and cytokines associated with mobilisation of EPCs[8 14 19] to the peripheral blood aiming at providing an insight into the humoral mechanisms that may control EPC pool derangement.

MATERIALS AND METHODS

Study subjects

Seventeen patients with CSX and 20 consecutive volunteers (referent population) were enrolled after providing informed consent according to the local institutional protocol. Criteria for inclusion of subjects with CSX were typical chest pain, normal 12-lead ECG at rest, a positive exercise ECG stress test response (>0.1 mV ST-segment depression at 80 ms after the J point in two or more contiguous leads) or an abnormal thallium scan with a normal coronary angiography. Echocardiography was performed in all patients for exclusion of valvular heart disease, impaired left ventricular function and myocardial hypertrophy. All patients with CSX were recruited by telephone at least 3 months after their previous episode of chest pain.

The referent population comprised 20 subjects preselected with a similar profile of risk factors for atherosclerosis and drug use as the CSX group. This was done to overcome the confounding effects on EPC numbers and functional properties.

Abbreviations: CRP, C reactive protein; CXS, cardiac syndrome X; EPC, endothelial progenitor cell; EPO, erythropoietin; HUVEC, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor

Table 1 Clinical profile of patients with CSX and the referent population

Clinical variables	CSX (n = 17)	Control (n = 20)	p Value
Gender, Male	29.4	30	0.988
Age (years), mean (SEM)	57.6 (2.7)	48.4 (4.4)	0.096
Smoking	35.7	10	0.195
Hyperlipidaemia	76.5	40	0.057
Hypertension	57.1	45	0.555
Drugs			
β Blockers	35.3	25	0.593
Calcium blockers	0	0	
ACE inhibitors	23.5	40	0.390
Nitrates	0	10	0.720
Statins	41.2	20	0.265
Diuretics	23.5	35	0.551

Results are shown as a percentage unless otherwise stated.

Assessment of EPC numbers by FACS

Heparinised blood (20 ml) was subjected to Ficoll gradient centrifugation (Amersham Sciences, Sweden) for recovery of peripheral blood mononuclear cells. Five million cells were stained for four-colour FACS analysis employing the following monoclonal antibodies: Cy-Q anti-CD45 (IQ products), phycoerythrin-anti-CD34 (IQ products), allophycocyanin-anti-VEGF-receptor 2 (KDR, R&D Systems) and FITC-anti-CD133 (R&D Systems, Germany). Staining was carried out immediately after recovery of the cells by Ficoll and each individual sample was read not later than 1 hour afterwards. Analysis was carried out using the same settings for all samples. The FACS results were represented as "number of events" per 1 million cells according to careful assessment of cell size. Each participant had two aliquots to ensure reproducibility, and we used the average. Results were included if intra-assay variability between the duplicate samples was <10%.

Proliferative and adhesive properties of EPCs

Peripheral blood mononuclear cells were isolated by Ficoll density gradient centrifugation assay. Adhesion assays were performed as previously reported.[17 19] Phenotypic confirmation of endothelial markers was performed as described.[17 19]

Matrigel tube formation assay

A Matrigel (Becton Dickinson, Germany) tube formation assay was performed on conditioned medium collected from patients with CSX and referent population. For this purpose, conditioned medium obtained from all subjects was collected on day 7 of culture and incubated in the presence of human umbilical vein endothelial cells (HUVEC; 10^5/well) for 36 hours, and tube scoring was performed by two independent observers according to the previously described method.[11 22] To overcome interassay and intra-assay variability we conducted the assay in triplicate and all samples were assayed on the same day. When deviation of >20% was evident between the two observers or when deviation was found within the triplicate samples, the test was repeated twice for two separate triplicate samples a week later with three control samples. The interassay variability was found to be <15%.

Assessment of erythropoietin (EPO) and vascular endothelial growth factor (VEGF) plasma levels by ELISA

EPO levels were determined by an ELISA kit (R&D Systems). Serum VEGF levels were determined by ELISA according to the manufacturer's instruction (R&D Systems). The VEGF level was calculated using a standard curve obtained with human recombinant VEGF-165 (from 15.6 to 2000 pg/ml). The inter-assay and intra-assay coefficients of variation were 9% and 11%, respectively.

Determination of high-sensitivity C reactive protein (CRP) serum concentrations

The assay for high-sensitivity CRP was conducted according to the manufacturer's instructions (Dade Behring Inc., Germany),

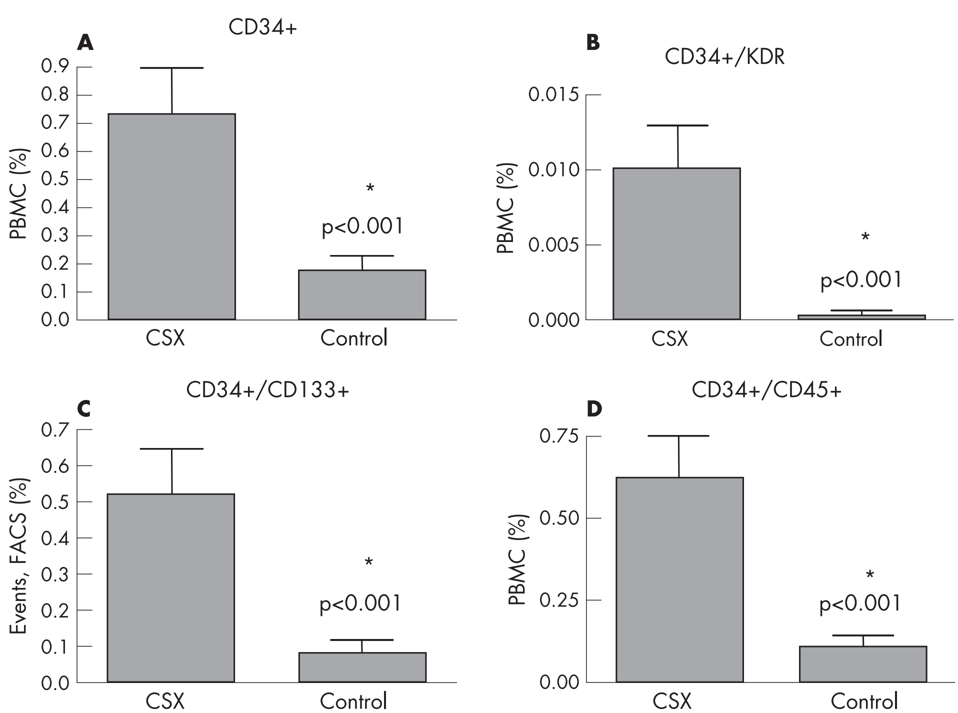

Figure 1 Assessment of circulating endothelial progenitor cells (EPCs) by FACS. FACS analysis was employed for assessment of circulating numbers of (A) CD34+; (B) CD34+/KDR; (C) CD34+/CD133+ and (D) CD34+/CD45+ with labelled antibodies as described in "Materials and methods". PBMC, peripheral blood mononuclear cells.

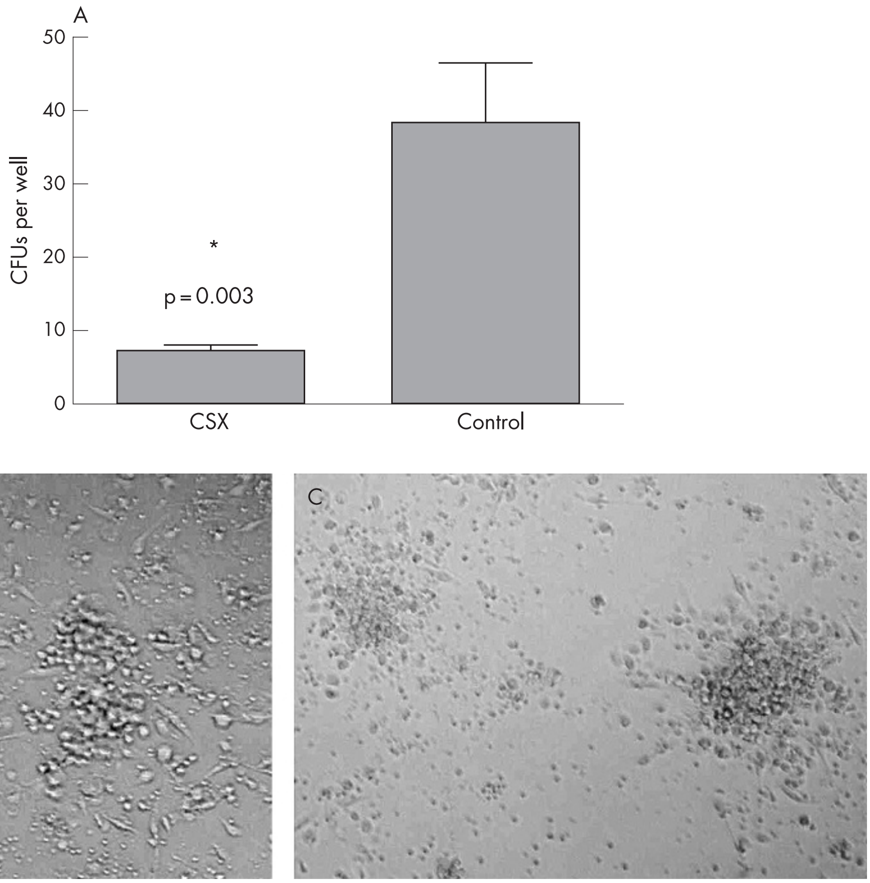

Figure 2 (A) Proliferative capacity of endothelial progenitor cells (EPCs) from patients with cardiac syndrome X (CSX) and the referent patients. A colony-forming unit (CFU) assay was employed at day 7 of culture for determination of the proliferative capacity of EPCs from patients with CSX and healthy subjects. A high-power field of EPCs from (B) a patient with CSX and (C) a healthy control.

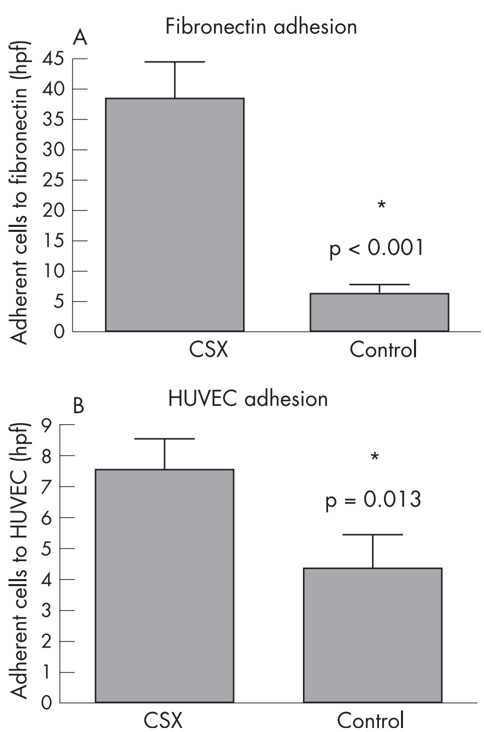

Figure 3 Adhesive properties of endothelial progenitor cells (EPCs) from patients with cardiac syndrome X (CSX) and healthy subjects. EPCs obtained after a 7-day culture of mononuclear cells on fibronectin-coated plates in the presence of endothelial cell medium were washed, labelled as described in ''Materials and methods'', and seeded on plates coated with (A) fibronectin or (B) cultured human umbilical vein endothelial cells (HUVEC) for determination of cell adherence.

with a sensitivity of 0.1 mg/l. The interassay and intra-assay coefficients of variation were 6% and 9%, respectively.

Determination of circulating adiponectin

Fasting serum adiponectin levels were measured using radioimmunoassay by Linco Research (St Charles, MO, USA); human adiponectin sensitivity is 1 mg/l on a 100 μl sample size. The assay range was 1–200 mg/l and the specificity <0.01%.

Statistical analysis

Categorical variables between groups were compared by the χ^2 test. A double-sided Student's *t* test was employed for comparison of EPC numbers and functional properties. Univariate correlations were made with Spearman's correlation coefficient. Multiple linear regression for the analysis of covariance was used to identify predictors of change in EPC counts and functional properties in a multivariate setting. A p value <0.05 was considered significant.

Power calculation for selection of the minimal number was assumed based on the following: type 1 error (α) = 5%; type 2 error (β) = 20% (power of 80%), range (ex.: CFU) = 90 ($\sigma = 15$) and magnitude of effect (d) = 15. Thus, $n = \{[(Z\alpha/2 - Z\beta)^{*}\sigma]/d\}^2 = 15$, yielding a minimum number of 15 subjects in each group.

RESULTS

As risk factors for atherosclerosis and drugs have been shown to influence the number of circulating EPCs, we matched both CSX and referent population for these variables. Table 1 shows the baseline clinical variables and the drugs used at the time of enrolment. Consistent with the literature on CSX, the majority of the patients were female. Mean weight of the patients with

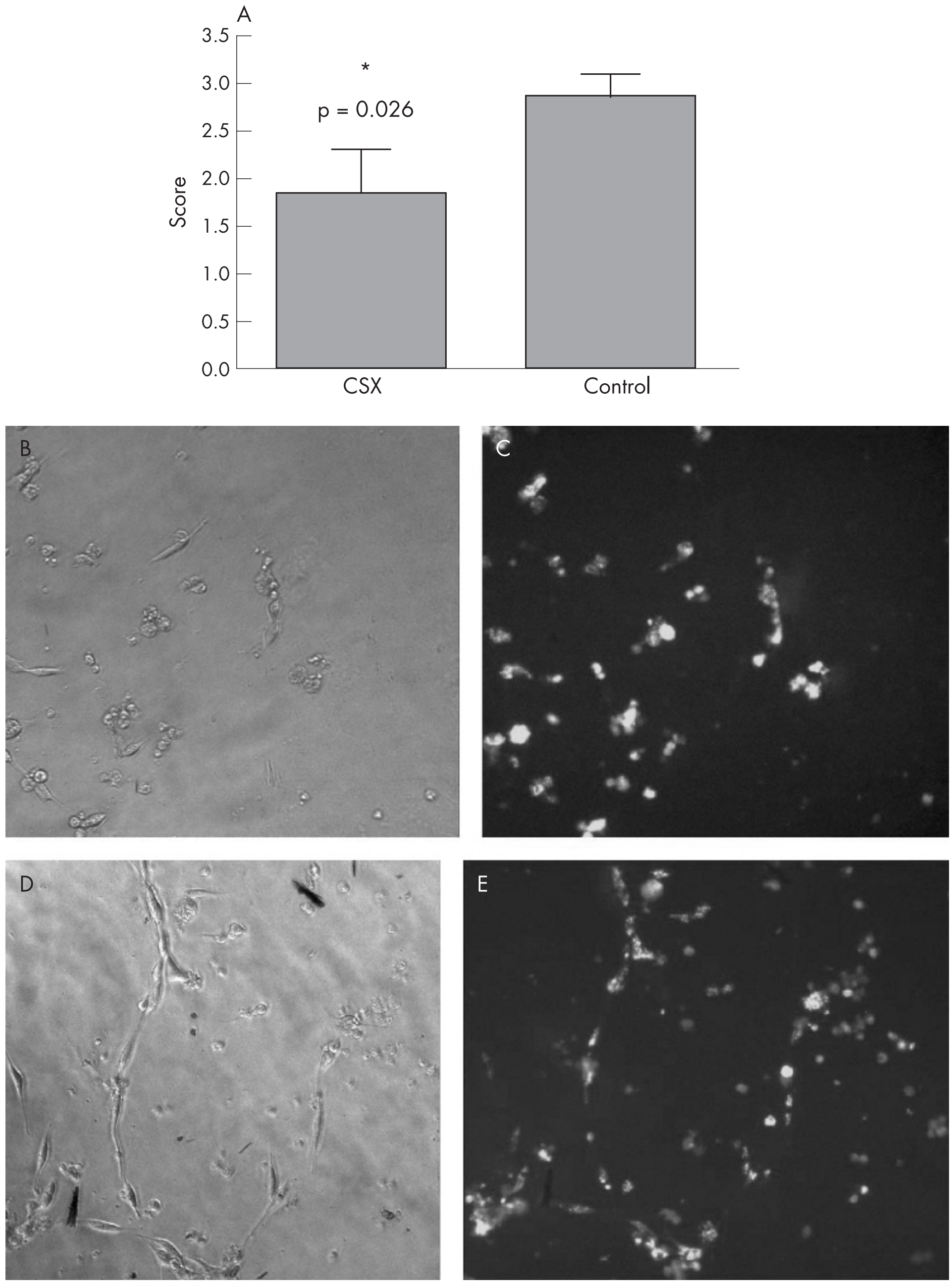

Figure 4 In vitro tube formation capacity of conditioned media from endothelial progenitor cells (EPCs) of patients with cardiac syndrome X (CSX) and healthy subjects. Conditioned medium of EPCs obtained from CSX and healthy subjects at day 7 was incubated in the presence of cultured human umbilical vein endothelial cells at a density of 10^5 cells/well for 36 hours. Scoring of triplicate samples was performed by two independent observers according to the standard method delineated in ''Materials and methods''. (A) Analysis of the scoring from all patients. (B) A representative phase contrast and (C) fluorescence labelled picture of endothelial cells incubated with conditioned medium obtained from EPCs of a patient with CSX, demonstrating the lack of assembly of endothelial cells in the presence of the cultured medium. (D) A representative phase contrast and (E) fluorescence labelled picture of endothelial cells incubated with medium obtained from cultured EPCs of a healthy subject.

CSX was 67 (9) kg as compared with 68 (7) kg in the referent population. None of the patients in either group was obese.

The number of circulating EPCs was assessed by FACS analysis. Intra-assay variability between duplicates did not exceed 15% and interassay variability was always $<20\%$. Patients with CSX had increased numbers of CD34+/KDR in comparison with the referent group (0.01% vs 0.0004%, $p<0.001$). A similar observation was evident for circulating CD34+/CD133+: a mean percentage of 0.52 in the patients with CSX vs 0.08 in the referent group ($p<0.001$). Interestingly, the total number of circulating CD34+ cells and the number of cells coexpressing CD34 and CD45 were also significantly increased in patients with CSX as compared with the referent group (fig 1). After age matching, differences remained significant in CSX as compared with controls for CD34+ (0.7 vs 0.21, $p = 0.005$), CD34+/KDR (0.009 vs 0.001, $p = 0.003$), CD34+/CD133+ (0.48 vs 0.09, $p = 0.004$) and CD34+/CD45+ (0.61 vs 0.13, $p = 0.005$).

No association was evident between any of the risk factors, age, gender or drugs and EPC numbers. Next, we compared the functional properties of EPCs obtained after replating and after a 7-day culture on fibronectin-coated plates in the presence of endothelial cell medium. The proliferative capacity of EPCs from patients with CSX was significantly impaired as compared

Table 2 Association between humoral markers and EPC number and properties in patients with cardiac syndrome X

Humoral markers	CRP r (p value)	EPO r (p value)	Adiponectin r (p value)	VEGF r (p value)
CFU	−0.27 (0.16)	−0.16 (0.31)	0.02 (0.47)	−0.11 (0.35)
Adherence to fibronectin	−0.09 (0.37)	−0.38 (0.1)	0.36 (0.1)	0.22 (0.21)
Adherence to HUVEC	−0.07 (0.4)	−0.51 (0.037)*	−0.12 (0.33)	0.35 (0.1)
CD34+/CD133+	−0.15 (0.3)	0.55 (0.026)*	0.11 (0.35)	−0.04 (0.44)
CD34+/VEGFR-2+ (KDR)	−0.16 (0.29)	0.2 (0.26)	−0.46 (0.043)*	0.52 (0.024)*

CFU, colony-forming unit; CRP, C-reactive protein; EPCs, endothelial progenitor cells; EPO, erythropoietin; HUVEC, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor2.
*$p<0.05$.

with the referent population as shown by the ability to form colonies (a mean of 6.9 per well vs 38.1 per well for patients with CSX and the referent group, respectively, $p = 0.003$; fig 2).

EPCs obtained from patients with CSX exhibited enhanced adhesiveness to matrix and mature endothelial cells. Accordingly, the mean number of cells adhering to fibronectin and HUVEC was 38.3 per higher power field (hpf) and 7.4/hpf ($p<0.001$) versus 6.3/hpf and 4.3/hpf, respectively ($p = 0.013$) (fig 3).

The ability of conditioned medium from CSX to support in vitro tube formation by the Matrigel assay was hampered as compared with the referent population (a mean score of 1.83 vs 2.85, respectively; $p = 0.026$)(fig 4).

To assess the relationship between cytokine levels and the number and properties of the peripheral EPC pool, we evaluated serum levels of selected mediators. VEGF serum levels correlated with the number of CD34+/KDR but not with the number of CD34+/CD133+ (table 2). EPO serum levels correlated significantly with the number of CD34+/CD133+ but not with the number of CD34+/KDR. Interestingly, a negative correlation was found between serum adiponectin levels and circulating numbers of CD34+/KDR (table 2). Yet, this correlation was irrespective of subject weight, which did not correlate with either EPC numbers or adiponectin levels.

DISCUSSION

It is well documented that patients with CSX show evidence of an enhanced inflammatory state and endothelial dysfunction.[1 2 23] Whether these findings have a causal role in the pathogenesis of this syndrome, or merely represent an epiphenomenon, is still unresolved. Regardless of the association between endothelial dysfunction and the occurrence of CSX, these patients have, other than typical chest pain, also functional evidence suggesting mismatch between coronary flow and myocardial demand.[3] We reasoned that if true ischaemia is present in patients with CSX, it should influence the pool of peripheral EPCs as has been documented for patients with ischaemic heart disease.[18–20]

Our initial observation was indeed suggestive of a significant mobilisation of bone marrow derived progenitors to the peripheral circulation (fig 1). This finding is evident by the significantly increased number of circulating CD34+, CD34+/KDR and CD34+/CD133+ cells. However, not only different populations of EPCs were mobilised, but also haematopoietic-derived progenitors coexpressing, along with CD34, also CD45 in the peripheral blood.

The EPC pool is influenced by multiple factors. Myocardial infarction[18] and ischaemia[19 20 24] have been shown to be associated with an increase in circulating EPC numbers, and VEGF has been incriminated in their mobilisation from the bone marrow to the peripheral blood. However, it seems that other stressful insults such as trauma are also associated with a shift in the EPC pool towards the periphery. Contrary to these findings, risk factors for atherosclerosis are associated with decreased numbers of circulating EPCs by mechanisms that are yet to be precisely resolved.[14 15]

In recent years, it has become apparent that functional properties of EPCs are important for the evolvement of impaired angiogenesis (reviewed by Urbich and Dimmeler[7] and Rafii and Lyden[8]). We have found that the ability of EPCs from patients with CSX to form endothelial-like colonies was significantly hampered, suggestive of a reduced proliferative state. This is also complemented by our finding that secreted products present in the conditioned media, cultured from EPCs of patients with CSX, were less supportive of the formation of tube-like structures in vitro. Collectively, these findings suggest that endothelial dysfunction documented in patients with CSX might be mediated by deranged functional properties of EPCs.

An additional function of EPCs relates to their ability to bind matrix and mature endothelial cells. Interestingly, we found that adhesiveness of EPCs from patients with CSX both to immobilised fibronectin and cultured HUVEC were significantly increased as compared with healthy subjects. Although apparently contradictory with the previously described functional properties, it may represent, similar to the rise in circulating EPC numbers, an attempt for compensation driven by secretion of humoral factors.

It has been suggested that several mediators have a role in the mobilisation and trafficking of EPCs from bone marrow into damaged tissues. VEGF[20 25] and EPO[26] have both been shown to be strong mobilisers of EPCs to the peripheral circulation. Interestingly, we found that whereas VEGF correlated with CD34+/KDR numbers, EPO levels correlated with CD34+/CD133+ cells. As CD133 is regarded as a marker of early EPCs that is lost during commitment to endothelial lineage,[27] it is possible that EPO and VEGF have differential effects on mobilisation of distinct subsets of EPCs. We have previously shown that CRP is associated with EPC numbers in patients with unstable angina.[19] In patients with CSX, we failed to detect a correlation between CRP levels and EPC numbers, suggesting that different factors may govern the mobilisation and homing of EPCs in different pathological states. An important limitation of the study is the small sample size. Patients with well-characterised CSX are difficult to find and compliance is problematic. The small numbers of patients could introduce statistical obstacles such as ''false positive'' results or, alternatively, a lack of correlation with cytokine levels.

We also evaluated levels of serum adiponectin, an adipokine with protective properties that is significantly diminished in patients with the metabolic syndrome[28] and has been shown to have antiangiogenic properties.[29] We found that adiponectin levels were negatively associated with the number of the more ''committed'' EPC subpopulation of CD34+/KDR cells, suggestive of a possible negative regulatory effect of this adipokine. These effects were irrespective of a possible association with the weight of the subject.

In conclusion, we have found that patients with CSX have significantly increased numbers of circulating EPCs that exhibit dysfunctional properties. These findings may explain the endothelial dysfunction in these patients and may help in the understanding of some aspects of the pathogenesis of this syndrome.

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